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Comparative Evaluation of Genetic Toxicity Patterns of Carcinogens and Noncarcinogens: Strategies for Predictive Use of Short-Term Assays

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The results of a recent comprehensive evaluation of the relationship between four measures of *in vitro* genetic toxicity and the capacity of the chemicals to induce neoplasia in rodents carry some important implications. The results showed that while the Salmonella mutagenesis assay detected only about half of the carcinogens as mutagens, the other three *in vitro* assays (mutagenesis in MOLY cells or induction of aberrations or SCEs in CHO cells) did not complement Salmonella since they failed to effectively discriminate between the carcinogens and noncarcinogens found negative in the Salmonella assay. The specificity of the Salmonella assay for this group of 73 chemicals was relatively high (only 4 of 29 noncarcinogens were positive). Therefore, we have begun to evaluate *in vivo* genetic toxicity assays for their ability to complement Salmonella in the identification of carcinogens.

Introduction and Design of Study

Long-term studies in rodents are the principal means by which potential human carcinogens are identified. The duration, costs, and concerns over the use of animals have been major problems associated with these rodent studies, and these concerns have provided the motivation to search for other experimental methods. The observed association between the ability of chemicals to induce both mutations and tumors served to promote development of the discipline of genetic toxicology (*1–5*). In the broad sense, *in vitro* genetic toxicity may be indicative of a chemical's capacity to heritably alter cellular phenotypes by any one of a number of potential mechanisms. A number of genetic toxicity assays have been used to identify chemicals that induce either gene or chromosomal mutations, or other effects, via direct interaction with DNA. Extensive efforts by the Environmental Protection Agency (EPA) to evaluate the capability of genetic toxicity assays to predict potential carcinogenicity (*6*) revealed two major impediments to a clear interpretation; first, insufficient results for chemicals that are noncarcinogens (*7*); and second, insufficient numbers of carcinogens and noncarcinogens that have been tested adequately in more than one or two *in vitro* genetic toxicity assays.

In order to address these inadequacies and to develop a data base that was amenable to systematic evaluation, the National Toxicology Program (NTP) initiated a project to provide genetic toxicity results for chemicals that were well characterized in rodents for carcinogenicity or noncarcinogenicity. Detailed results of this evaluation have been reported (*8*), and only certain aspects will be emphasized in this report. The chemicals or substances selected for evaluation were chosen strictly because they had been assayed for carcinogenicity in rodents under the aegis of the NTP (*9*). In order to be included, the carcinogenicity studies must have been of 2-year duration, including both rats and mice, and completed between December 1976 and January 1985. Initially, 83 chemicals were selected by these criteria; however, some substances tested during this period such as gilsonite, guar gum, agar, gum acacia, propylene, etc., could not be tested adequately *in vitro* due to their physical state or other reasons, and were, therefore, excluded from further consideration. Our evaluations were made using the 73 chemicals listed in Table 1.

The *in vitro* and *in vivo* assays that provided genetic toxicity patterns were performed on chemicals, under code, in a number of laboratories. The assay protocols

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Table 1. Rodent carcinogenicity and STT results for 73 chemicals.

Chemical	Carcinogenicity[a]				Short-term tests[b]			
	MR	FR	MM	FM	SAL	MOLY	ABS	SCE
Benzene	+	+	+	+	−/−	−/−	−/−	+/−
3-Chloro-2-methylpropene	+	+	+	+	−/−	+/0	+/+	+/+
Di (2-ethylhexyl) phthalate	+	+	+	+	−/−	−/−	−/−	+/−
1,2-Dibromo-3-chloropropane	+	+	+	+	−/+	+/0	+/+	+/+
1,2-Dibromoethane	+	+	+	+	−/+	+/+	+/+	+/+
Diglycidyl resorcinol ether	+	+	+	+	+/+	+/0	+/+	+/+
Ethyl acrylate	+	+	+	+	−/−	+/0	−/+	−/+
4,4′-Methylenedianiline 2HCl	+	+	+	+	−/+	+/0	+/+	+/+
4,4′-Oxydianiline	+	+	+	+	+/+	+/0	+/+	+/+
Polybrominated biphenyl mixture	+	+	+	+	−/−	−/−	−/−	−/−
Propylene oxide	+	+	+	+	+/+	+/0	+/+	+/+
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	+	+	+	+	−/−	−/−	−/−	−/−
1,3-Dichloropropene	+	+	I	+	+/+	+/0	−/−	+/+
Cinnamyl anthranilate	+	−	+	+	−/−	−/+	−/−	−/−
HC Blue 1	E	+	+	+	+/+	+/0	−/+	+/+
Reserpine	+	−	+	+	−/−	−/−	−/−	−/−
Selenium sulfide	+	+	−	+	+/+	+/0	+/+	+/−
2,4- and 2,6-Toluene diisocyanate	+	+	−	+	−/+	+/+	+/−	+/−
Allyl isovalerate	+	−	−	+	−/−	+/0	−/+	+/+
Benzyl acetate	E	−	+	+	−/−	+/0	−/−	−/−
bis (2-Chloro-1-methylethyl) ether	−	−	+	+	−/+	+/0	−/+	+/+
C.I. Disperse Yellow 3	+	−	−	+	+/+	−/+	−/−	+/−
C.I. Solvent Yellow 14	+	+	−	−	−/+	−/+	−/−	+/+
Cytembena	+	+	−	−	+/+	+/0	+/+	+/+
1,2-Dichloropropane	−	E	+	+	+/−	−/+	+/+	+/+
2,6-Dichloro-*p*-phenylenediamine	−	−	+	+	−/+	+/0	+/+	+/+
Di(2-ethylhexyl) adipate	−	−	+	+	−/−	−/−	+/−	E
Dimethyl morpholinophosphoramidate	+	+	−	−	−/−	+/0	+/−	+/−
Pentachloroethane	E	−	+	+	−/−	+/0	−/−	+/−
1,1,1,2-tetrachloroethane	E	−	+	+	−/−	−/+	−/−	+/−
Trichloroethylene	I	−	+	+	−/−	−/+	−/−	+/+
Zearalenone	−	−	+	+	−/−	−/−	+/−	+/−
Allyl isothiocyanate	+	E	−	−	E	+/0	+/+	−/+
11-Aminoundecanoic acid	+	−	E	−	−/−	−/−	−/−	+/−
2-Biphenylamine HCl	−	−	−	+	−/+	−/+	+/−	−/−
Butyl benzyl phthalate	I	+	−	−	−/−	−/−	−/−	−/−
Chlorodibromomethane	−	−	E	+	−/−	+/0	−/−	−/+
D & C Red 9	+	E	−	−	+/−	−/−	−/−	−/−
Dimethyl hydrogen phosphite	+	E	−	−	−/+	−/+	+/+	+/+
Isophorone	+	−	E	−	−/−	+/0	−/−	+/−
Melamine	+	−	−	−	−/−	−/−	−/−	E
Monuron	+	−	−	−	−/−	E	−/+	+/+
Tris(2-ethylhexyl)phosphate	E	−	−	+	−/−	−/−	−/−	−/−
Ziram	+	−	−	E	+/+	+/0	+/+	−/−
L-Ascorbic acid	−	−	−	−	E	E	−/−	+/−
Benzoin	−	−	−	−	E	−/+	−/−	E
Bisphenol A	E	−	−	−	−/−	−/−	−/−	E
C.I. Acid Orange 10	−	−	−	−	−/−	−/−	−/+	−/−
C.I. Acid Red 14	−	−	−	−	−/−	−/−	−/−	E
C.I. Acid Yellow 73	E	−	−	−	−/−	+/0	−/−	+/+
Caprolactam	−	−	−	−	−/−	−/−	−/−	−/−
Chlorobenzene	E	−	−	−	−/−	+/0	−/−	+/−
2-Chloroethanol	−	−	−	−	−/+	−/+	+/+	+/+
Diallyl phthalate	−	E	E	E	−/−	+/+	−/+	−/+
1,2-Dichlorobenzene	−	−	−	−	−/−	−/+	−/−	−/+
Dimethyl terephthalate	−	−	E	−	−/−	−/−	−/−	−/−
Ethoxylated dodecyl alcohol	−	−	−	−	−/−	−/−	−/−	−/−
Eugenol	−	−	E	E	−/−	+/0	−/+	+/+
FD & C Yellow No. 6	−	−	−	−	−/−	−/+	−/−	E
Geranyl acetate	−	−	−	−	−/−	−/+	−/−	+/+
Hamamelis water (witch hazel)	−	−	−	−	−/−	−/−	−/−	−/−

Table continued next page

Table 1. *Continued*

Chemical	Carcinogenicity[a]				Short-term tests[b]			
	MR	FR	MM	FM	SAL	MOLY	ABS	SCE
HC Blue 2	–	–	–	–	+/+	+/0	–/–	+/+
8-hydroxyquinoline	–	–	–	–	–/+	+/0	–/+	+/+
Malaoxon	–	–	–	–	–/–	+/0	–/–	+/+
D-Mannitol	–	–	–	–	–/–	–/–	–/–	–/–
DL-Menthol	–	–	–	–	–/–	–/–	–/–	–/–
Phenol	–	–	–	–	–/–	–/+	–/+	+/+
Propyl gallate	E	–	E	–	–/–	+/0	+/–	+/+
Sodium (2-ethylhexyl) alcohol sulfate	–	–	–	E	–/–	–/–	–/–	–/–
Stannous chloride	E	–	–	–	–/–	–/–	+/+	+/+
Sulfisoxazole	–	–	–	–	–/–	+/0	–/–	+/+
Titanium dioxide	–	–	–	–	–/–	–/–	–/–	–/–
2,6-Toluenediamine 2HCl	–	–	–	–	–/+	+/0	+/–	+/+

[a] MR, male rat; FR, female rat; MM, male mouse; FM, female mouse; E, equivocal; I, incomplete.
[b] (–/–) Activity without S9/activity with S9; (/0) assay not performed.

for each of the four *in vitro* assay systems had been developed previously (*10–12*) in order to ensure intra- and interlaboratory reproducibility and subjected to validation using chemicals tested under code in order to ensure objective results (*10–13*). Assay protocols for the other short-term tests (STT) (Table 2) in which subsets of the 73 chemicals were tested have been developed to achieve similar standards of performance. Aliquots of the 73 chemicals were shipped from a central repository, under code, to the testing laboratories. Where it was possible, the same chemical lot that was used in the rodent carcinogenicity studies was also tested by the laboratories conducting the genetic toxicity assays. The criteria that define an acceptable assay have been reported previously for each test system (*10–13*). Conclusions regarding the rodent carcinogenicity of the chemicals were taken directly from the NTP technical reports. The NTP currently uses a "categories-of-evidence" scheme in which, for each sex/species group, the carcinogenic response is classified into one of five categories. There are two categories of positive response ("some" or "clear"), one for equivocal responses, a negative category, and one category for studies that are inadequate for evaluation. For the purpose of this evaluation, chemicals that exhibited equivocal activity in animal studies were included in the category of noncarcinogens. Chemicals that showed equivocal activity in the STT were also included in the negative category.

Rodent Carcinogenicity Results

The tumorigenicity patterns for the 44 carcinogens (shown in Table 1) are quite variable in sex, species, site and frequency, and include 12 chemicals that were positive in only one of the four sex/species combinations. Twenty of the substances were judged to demonstrate no evidence of carcinogenicity, and nine of the studies were judged equivocal. Each of the studies included an equal number (50) of treated and vehicle control animals in each sex/species group. The control animals were also subject to complete postmortem examination. The results of the control group evaluations have been compiled to establish the spontaneous tumor incidence patterns for each sex/species (*14*).

The most frequent sites of tumor induction were the liver (24/44 chemicals), lung (8 chemicals), forestomach (6 chemicals), thyroid (5 chemicals), and kidney (2 chemicals) (*8*). The liver, lung, and thyroid show spontaneous tumors at frequencies of 10 to 30% in untreated control groups (called common tumors), whereas spontaneous tumors are rarely seen in the forestomach or kidney (uncommon tumors). Liver tumors were the only tumors induced by 12 carcinogens, 1 of these produced liver tumors in rats only, 8 in mice only, 1 in at least one sex of both species, and 2 chemicals induced only liver tumors in all four sex/species groups. Nine chemicals induced liver tumors in addition to common tumors at other sites; 4 chemicals induced liver tumors in combination with uncommon tumors in at least one other site. Nine of the rodent carcinogens that did not induce liver tumors induced common tumors at other organ sites.

Five chemicals induced only uncommon tumors, 3 of which (administered by gavage) induced only papillomas and/or carcinomas of the forestomach in both sex/species. Endocrine tumors, e.g., follicular cell tumors of the thyroid, adrenal gland, pancreatic ascinar cells, pheochromocytomas of the pituitary, were induced by 9 chemicals. Five of these induced thyroid follicular cell adenomas or carcinomas.

The sex/species distribution of tumors induced by the 44 carcinogens revealed that 18 chemicals induced tumors in at least one sex of both species, and 12 of these induced tumors in both sexes of both species. Eight of these 12 trans-sex/species carcinogens induced tumors at multiple sites in at least one sex/species. Also, 7 of the trans-sex/species carcinogens induced uncommon tumors, while 2 induced tumors only in the liver. Twenty-four of the 44 chemicals induced tumors in only one species (12 each in rats or mice). Eight (of the 12) induced tumors in only male rats, but none of the chemicals induced tumors only in male mice, and only 1 chemical induced tumors only in the female rat. Liver tumors

Table 2. STT results for selected chemicals.

Chemical	DL (SLRL)	*In vivo* ABS	*In vivo* SCE	*In vivo/ in vitro* UDS	*In vitro* UDS	BALB/c	SHE	SHE/SA7	Rat RLV
Allyl isothiocyanate	−					−			
Allyl isovalerate	−								
11-Aminoudecanoic acid	−			−	−	+			
L-Ascorbic acid					−	−	−	−	+
Benzene						+			
Benzoin		−	−		−	−	E[a]	+	E
Benzyl acetate				−	−	+			
2-Biphenylamine HCl	−	−	−		−	−			
Bis(2-chloro-1-methylethyl) ether	?			−	−				
Bisphenol A					−	−	−	+	+
Butyl benzyl phthalate	−								
C.I. Acid Orange 10					−	+			
C.I. Acid Yellow 73	−				−				
C.I. Disperse Yellow 3					+				
C.I. Solvent Yellow 14				−	+	+			
Caprolactam		−	−		−	+	+	E	−
Chlorodibromomethane		−	−		−				
2-Chloroethanol	−	−	−		−				
Cinnamyl anthranilate				−	−	+	+	+	−
Cytembena	−				−				
D & C Red 9					−	+			
Di(2-ethylhexyl) adipate	−								
Di(2-ethylhexyl) phthalate	−	−	−	−	−	−	+	E	+
Diallyl phthalate	−								
1,2-Dibromo-3-chloropropane	+	−	+						
1,2-Dibromoethane					+				
2,6-Dichloro-*p*-phenylenediamine				−	−	+	+	+	+
1,2-Dichlorobenzene		−	+/−						
1,2-Dichloropropane	−								
1,3-Dichloropropene	+								
Diglycidyl resorcinol ether	+	+	+		−	+			
Dimethyl hydrogen phosphite	−				−				
Dimethyl morpholinophosphoramidate	+								
Ethyl acrylate	−								
Eugenol	−								
FD & C Yellow No. 6					−	+			
Geranyl acetate					−	−	+	+	+
Hamamelis water (witch hazel)	−								
HC Blue 1	?	+	+	−	+	+	+	+	+
HC Blue 2	−	−	+	−	+	−	−	−	+
8-Hydroxyquinoline	?	−	−		−	−			
Isophorone					−				
D-Mannitol	−								
Melamine					−	−			
4,4-Methylenedianiline 2HCl		−	+	−	+	+			
Monuron					−				
4,4′-Oxydianiline		+	−	−	−	+	+	+	+
Pentachloroethane					−				
Phenol	−	+	+/−						
Polybrominated biphenyl mixture				−	−	−			
Reserpine	?			−	−	+	+	+	+
Selenium sulfide				−	+				
Sodium (2-ethylhexyl)alcohol sulfate	−								
Stannous chloride					−				
Sulfisoxazole					−				
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	−								
Titanium dioxide					−				
2,4- and 2,6-Toluene diisocyanate					−				
2,6-Toluenediamine 2HCl		−	+						
Trichloroethylene	?			−	−	−			
Tris(2-ethylhexyl) phosphate					−				
Zearalenone					−				
Ziram					−				

[a] E, equivocal

were the only tumors induced by 8 of the 12 chemicals that were carcinogenic only in mice.

Some sites of tumor induction are related to route of exposure (e.g., nasal or glossal tumors were seen only in inhalation studies, and forestomach tumors only in gavage studies). The 3 chemicals administered by inhalation (1,2-dibromo-3-chloropropane, 1,2-dibromoethane and propylene oxide) were tumorigenic in all four sex/species groups. The thyroid or urinary bladder were the only tumor sites for four of the chemicals inducing tumors in a single sex of one species.

Evaluation of STT

The 73 chemicals in this evaluation were tested in each of the four principal *in vitro* assay systems; i.e., Salmonella/microsome mutagenicity assay (SAL); mouse L5178Y lymphoma $TK^{+/-}$ forward mutation assay (MOLY); chromosome aberrations assay (ABS) in Chinese hamster ovary (CHO) cells; and the assay for sister chromatid exchange (SCE) in CHO cells. These assays are representative of the most widely used methods for characterizing mutagenicity and clastogenicity. With the exception of the MOLY assay, chemicals were routinely tested in these four end points with and without Aroclor 1254-induced rodent liver S9 preparations. If a chemical exhibited activity in the MOLY assay in the absence of S9, it was usually not tested with S9.

Subsets of the 73 chemicals were also evaluated in several other STTs: in the *in vitro* unscheduled DNA synthesis (UDS) in rat hepatocytes assay; in four *in vitro* mammalian cell transformation systems (Table 2); in the *in vivo* sex-linked recessive lethal mutagenicity assay in Drosophila; in the *in vivo-in vitro* unscheduled DNA synthesis (UDS) and scheduled DNA synthesis (S-phase) assays in rodent hepatocytes; and in the *in vivo* cytogenetics assays for chromosome aberrations (ABS) and sister chromatid exchanges (SCE) in mouse bone marrow.

For the purpose of calculating sensitivity, specificity, positive and negative predictivity, and overall concordance values (Table 3), an equivocal response in the short-term tests was considered to be negative.

Mutagenicity in *Salmonella typhimurium*

Chemicals were evaluated in four to five strains of *Salmonella typhimurium* using microsomal (S9) fractions from rat or hamster livers as the exogenous metabolic activation system. The assay protocol and the criteria for evaluating responses have been published elsewhere (*10*). Clear evidence of mutagenicity is indicated by a reproducible, dose-related increase in the mutant yield from the chemical-treated group compared to the solvent control group in any one Salmonella strain with or without exogenous metabolic activation.

Twenty-four of the 73 chemicals were mutagenic in Salmonella, and 22/24 were positive in two or more strains. While the sensitivity of the Salmonella assay was low (45%) (Table 3), only 20 of the 44 carcinogens were detected, the positive predictivity was high; 20 of the 24 chemicals mutagenic in Salmonella were rodent carcinogens. Only 4 of the 12 chemicals that induced only liver tumors were mutagenic in Salmonella, as were 4 of the 12 chemicals inducing tumors in a single sex/species. Twelve of the 24 Salmonella mutagens required metabolic activation in order to demonstrate a positive effect.

Table 3. Characteristics of four STT for predicting carcinogenicity.

	SAL		MOLY		ABS		SCE	
	+	−	+	−	+	−	+	−
Rodent								
+	20	24	31	13	24	20	32	12
−	4	25	16	13	9	20	16	13
Sensitivity[a]	45		70		55		73	
Specificity[b]	86		45		69		45	
Positive predictivity[c]	83		66		73		67	
Negative predictivity[d]	51		50		50		52	
Concordance[e]	62		60		60		62	

[a] Percent of carcinogens yielding a positive STT result.
[b] Percent of noncarcinogens yielding a positive STT result.
[c] Percent of STT positives that are carcinogens.
[d] Percent of STT positives that are noncarcinogens.
[e] Percent of qualitative agreements between STT and rodent carcinogenicity test results.

The overall concordance of the mutagenic response in any Salmonella strain with the rodent carcinogenesis response is 62% (Table 3). However, the agreement between mutagenicity in Salmonella and tumorigenesis in any one of the four sex/species (i.e., the positive predictivity of the assay) is 83%. These results show that mutagenicity in Salmonella, obtained under the protocols used in this study and evaluated according to the stated criteria, indicates a high probability, though not a certainty, for tumorigenicity in rodents. Conversely, the absence of a mutagenic response in any of the Salmonella strains is not predictive of nontumorigenicity.

Mutagenicity in Mouse Lymphoma (L5178Y) Cells (MOLY)

Of the 44 carcinogens, 31 (sensitivity = 70%) (Table 3) were positive in the MOLY assay based upon the published evaluation criteria (*12,13*). However, the assay also detected mutagenic activity in 16 (55%) of the 29 noncarcinogenic or equivocal substances. Thirteen of the carcinogens and 13 noncarcinogens exhibited no mutagenic activity in MOLY cells. The range of active doses varied from 0.0625 mg/mL for ziram to 1700 mg/mL for dimethyl hydrogen phosphate, but the lowest positive dose (LPD) did not show any relationship with the LPD for carcinogenicity in rodents or sex/species distribution of carcinogenic effects. There was a total of 47 (of the 73) chemicals that showed a positive effect in MOLY cells; the overall concordance of the assay with carcinogenicity was 60% (Table 2). In comparison to the Salmonella mutagenesis results, all but 1 (D&C Red 9) of the 24 chemicals mutagenic in Salmonella were mu-

tagenic in MOLY cells. In addition, positive responses were induced in MOLY cells by 24 chemicals (12 carcinogens) that were not mutagenic in the Salmonella assay (Table 2). Thus, although the results reflect a higher sensitivity (70%) than that of the SAL assay, the specificity of the MOLY assay was only 45% (Table 3) (*8*). These and other results (*8*) suggest that the MOLY assay cannot serve in a complementary role to the Salmonella assay. Since the specificity of the assay indicates a high rate of false positive results in this data set, some additional studies are necessary to determine if chemicals that show a Salmonella negative, mouse lymphoma positive result possess intrinsic mutagenic potential not detected in the Salmonella assay or whether these are idiosyncratic responses of the MOLY cells to some other properties of the chemicals.

Chromosomal Aberrations (ABS) and Sister Chromatid Exchange (SCE) *Induction in Chinese Hamster Ovary* (CHO) Cells

Chromosomal aberrations were induced by 33/73 chemicals (24/44 carcinogens and 9/29 noncarcinogens). Twenty carcinogens demonstrated no activity in this assay. The positive and negative predictivity of this assay for these 73 chemicals is 73% (24/33) and 50% (20/40), respectively (Table 3). The addition of an S9 fraction was required in order to demonstrate a response for 10 of the 33 positive chemicals. Dependence or independence of exogenous metabolic activation did not appear to be related to sex/species or number of sites of tumors induced by carcinogens. The 9 noncarcinogens (8-hydroxyquinoline, diallyl phthalate, eugenol, C.I. Acid Orange 10, phenol, propyl gallate, 2,6-toluenediamine, stannous chloride, and chloroethanol) did not demonstrate any clear differences in potency or patterns, i.e., simple or complex aberrations, of clastogenesis when compared with the carcinogens.

In relation to the SAL mutagenesis results, 4 of the 20 rodent carcinogens that were SAL positive did not induce ABS. Of the 24 rodent carcinogens inactive in the SAL assay, only 8 induced aberrations in CHO cells. These results do not strongly recommend the chromosome aberration assay as being complementary to the SAL assay. Nine of the rodent noncarcinogens induced aberrations (Table 3), and 3 of these were chemicals that were also mutagenic in the SAL, MOLY, and SCE assays. Only 1 chemical, C.I. Acid Orange 10, induced ABS as the only positive response among the four major STT (Table 1).

SCEs were induced by 32 of the 44 carcinogens; conversely, 16 of 29 noncarcinogens also induced SCEs (Table 3). There were 3 carcinogens (benzene, DEHP, 11-aminoundecanoic acid) for which SCE induction was the only observed *in vitro* positive response; in all three instances the chemical was active in the absence of S9. In relation to the other three end points, the sensitivity (73%) of the CHO/SCE assay was most similar to MOLY mutagenesis (70%) compared to Salmonella (45%) or CHO/ABS (55%) responses (Table 3). However, while the sensitivity of the CHO/SCE and MOLY assays were high, they both exhibited the lowest specificity (45%) (Table 3). These assays alone or in combination do not provide a complementary assay for the Salmonella assay (*8*).

The results obtained with the four assays described above did not show significant differences in individual concordance with the rodent carcinogenicity results since all of the end points showed approximately 60% accuracy (Table 3). Further, there was no evidence of complementarity among the four assays and no battery or series of tests constructed from these end points improved substantially on the overall performance of the Salmonella assay. However, since such a large number of carcinogens were negative in all, or positive in only one or two assays, there is a need to seek one or more assays that may complement, for example, the Salmonella assay. Complementarity could be demonstrated by the detection of more carcinogens without a concomitant increase in the number of noncarcinogens detected as genotoxic. It seems reasonable to search among available short-term *in vivo* assays for one or more end points that can improve on the sensitivity of the *in vitro* assays without having a negative effect on the specificity (i.e., increasing the number of false positives). We have begun to test the same 73 chemicals in some *in vivo* assays for which we have developed protocols that generate comparably reproducible results. While only a subset of chemicals have been tested thus far, and additional tests are currently in progress, the results available to date are included in Table 2. This table also presents limited results obtained with some other *in vitro* assays.

Mutagenicity in Drosophila

Twenty-six of the 73 chemicals were treated for mutagenicity in the sex-linked recessive lethal mutation assay in *Drosophila melanogaster* (*15*) (Table 2). Only 4 chemicals induced mutations and all of these were rodent carcinogens. Fifteen other rodent carcinogens did not induce mutations in Drosophila. Thus, while the positive predictivity and specificity of the assay were high, the sensitivity of the assay is low. Three of the 4 chemicals positive in Drosophila were also mutagenic in Salmonella, but 9/26 other chemicals that were Salmonella mutagens did not induce mutations in Drosophila. Similarly, these 4 Drosophila mutagens were active in the other STTs. However, the Drosophila assay is an *in vivo* germ cell mutagenesis assay where effects are observed only in the progeny and, therefore, may not be appropriate as a predictor of carcinogenicity.

In Vivo Cytogenetic Effects—Mouse Bone Marrow

Mouse bone marrow cytogenetics results are currently available for only 16 of the 73 chemicals for effects

on chromosome aberrations or sister chromatid exchange induction in B6C3F1 mice (*17*) (Table 2). Eight of these chemicals demonstrated carcinogenic activity, and 8 were noncarcinogenic. If an effect on either end point (ABS or SCE) is considered, 7 of the carcinogens and 4 of the noncarcinogens were correctly identified (accuracy = 69%). Six of the 8 carcinogens and 4 of the 8 noncarcinogens induced SCEs (accuracy = 63%). The only carcinogen not identified by either assay was di(2-ethylhexyl)phthalate (DEHP). With the exception of caprolactam, the other noncarcinogens demonstrated evidence of *in vitro* genetic toxicity, although 1,2-dichlorobenzene and benzoin were positive only in the MOLY assay and CHO/SCE end points.

Two chemicals showing clear evidence of *in vitro* genetic toxicity but that were not rodent carcinogens (2-chloroethanol and 8-hydroxyquinoline) did not induce cytogenetic effects *in vivo*, suggesting that *in vivo* cytogenetics assay might serve to discriminate between *in vitro* genotoxic carcinogens and noncarcinogens that exhibit *in vitro* genetic toxicity. Phenol induced cytogenetic effects both *in vitro* and *in vivo* but was not tumorigenic in rodents.

Unscheduled DNA Synthesis (UDS) *In Vitro*

The unscheduled DNA synthesis (UDS) in rat (F344) liver hepatocytes assay (*16*) was performed on 44 chemicals (Table 2). The assay detected 6 of the 30 carcinogens and 1 (H.C. Blue 2) of the 14 noncarcinogens induced a positive response. Twenty of the 30 carcinogens induced liver tumors in either rats or mice. The assay detected 4 (4,4′-methylenedianiline·2 HCl, C.I. Disperse Yellow 3, C.I. Solvent Yellow 14, and selenium sulfide) of the 9 carcinogens that induced hepatic tumors in rats and only 1 (H.C. Blue 1) of the 10 mouse hepatocarcinogens. The sixth rodent carcinogen detected was 1,2-dibromoethane, which induced tumors at multiple sites in both sex/species, but did not induce liver tumors. The 5 hepatocarcinogens that were detected are either primary amines or capable of being converted into primary amines; however, 6 other carcinogens that are primary amines did not induce a positive response. The 6 carcinogens positive in the UDS assay were also positive in at least three other *in vitro* end points, but 9 other carcinogens that induced responses in at least three end points did not induce a response in the UDS assay.

In Vivo–In Vitro UDS

Fifteen chemicals have been evaluated in the UDS assay that measures unscheduled DNA synthesis in cultured hepatocytes derived from animals exposed to test chemicals (*18*) (Table 2). All except H.C. Blue 2 were rodent carcinogens and 13 of the 14 induced liver tumors in either or both rodent species. None of the chemicals induced a UDS response, even though 12 of the 13 hepatocarcinogens were tested in the same sex/species in which the chemicals were tumorigenic. The same 15 chemicals were also tested in the *in vitro* UDS assay, and with the exception of 4 (4,4′-methylenedianiline·2HCl, H.C. Blue 1, H.C. Blue 2, and selenium sulfide) did not induce *in vitro* unscheduled DNA synthesis.

Mammalian Cell Transformation *In Vitro*

Four different mammalian cell transformation assays have been evaluated by the NTP (*19–22*), but only 12 chemicals from the group of 73 have been tested in all four systems (Table 2). All 6 of the carcinogens tested induced positive responses, and with the exception of DEHP, they were active in all four systems. Transformation was the only *in vitro* STT end point for which the carcinogen reserpine induced a positive response. However, the 6 noncarcinogens tested also induced a response in at least one system. The data are insufficient to adequately evaluate these assays at this time and more noncarcinogens and nongenotoxic carcinogens need to be included for comparative evaluation.

Discussion

While many unanswered questions remain, the results of this evaluation carry several important implications for the use of short-term tests to identify potential carcinogens. The overall concordance among results in the four principal genetic toxicity tests shows that the assays can be used reliably to identify genotoxic chemicals. For 59/73 (81%) of the chemicals, the genetic toxicity results were in agreement for 4/4 or 3/4 assays used. For 34 of the 59 (58%), all four end points were in agreement. While conclusions based on the limited number of chemicals tested in *in vivo* systems must be considered preliminary, the presence or absence of an *in vivo* effect appears to be highly informative. The limited use of short-term, whole animal assays is necessary, particularly for chemicals that fail to induce effects *in vitro*, e.g., benzene, or where the substance cannot be adequately metabolized or converted to the same DNA or chromatin-reactive intermediates that may be generated in the whole animal. Conversely, it may also be necessary to utilize *in vivo* assays for chemicals that show clear evidence of genetic toxicity *in vitro*, since disposition or metabolism of chemicals *in vivo* can also mitigate against the induction of observable genetic toxicity *in vivo*.

The choice of the available and well-characterized *in vitro* short-term test systems depends upon the specific answers needed. If the primary goal is the prospective identification of potential carcinogens, the Salmonella mutagenesis assay has some advantages over the others in reproducibility and reliability of technical execution, relatively low cost, and short duration. The evaluation of this data set of 73 indicates that none of the other *in vitro* end points, either singly or in combination increase the overall concordance with the rodent results to a degree significantly greater than the Salmonella assay

alone. While the Salmonella assay produced only a single positive response that was discordant with the other STT assays, there were 23/73 chemicals that were negative in Salmonella and positive in at least two of the other end points; 11 of these 23 results were discordant with the MOLY mutagenesis and CHO/SCE end points, and 6 of the 11 were not rodent carcinogens.

Our evaluation, therefore, suggests that the potential genetic toxicity of most chemicals can be detected using the Salmonella mutagenesis assay. No other single *in vitro* assay appears to be sufficiently complementary to SAL, to the point where it is cost effective to perform the additional assay. However, several of the assays, e.g., MOLY or CHO/ABS or SCE, could be useful in a confirmatory capacity. A conservative *in vitro* assay such as the UDS assay or the *in vivo* Drosophila recessive lethal assay can also prove useful for confirmatory purposes, since a false positive response in these assays is very unusual. The verification of the actual value of the *in vivo* assays and the final choice of assays must await the results of testing the remainder of the 73 chemicals in these systems.

Therefore, it appears that clear evidence of mutagenicity, that is, a statistically significant effect in at least one strain of Salmonella, indicates a high probability of potential tumorigenicity in rodents. It is not, however, a certain indicator of tumorigenicity, since 4 chemicals that were not carcinogenic were mutagenic in Salmonella (8-hydroxyquinoline, 2-chloroethanol, 2,6-toluenediamine, and H.C. Blue 2). However, since these 4 chemicals were also active in three other *in vitro* end points, these are excellent candidates for future in-depth animal and *in vitro* studies, in which chemical disposition, pharmacokinetics, extent of DNA binding, identification of DNA adducts, etc., can be elucidated.

Conversely, the absence of evidence of genetic toxicity in SAL or other *in vitro* tests does not carry any clear implications as to the probable noncarcinogenicity of the chemical. Six substances that induce neoplasia in rodents did not induce any response in four end points (SAL, MOLY, CHO/SCE, and ABS), nor did 10 noncarcinogens. Therefore, prospectively, such chemicals could not be distinguished on the basis of any of the tests used in this study.

The classification of rodent tumorigenicity is expressed on an individual sex/species basis as either clear, some, or no evidence of carcinogenic activity. This distinction is based principally on the statistical magnitude of the response in the individual groups. For the purpose of this evaluation, the sex/species patterns, tumor sites and types, and malignancy were also considered. Twelve of the 44 carcinogens were carcinogenic in both sexes of both species and 8 of the 12 induced responses in at least three *in vitro* systems. Of the remaining 4, benzene induced chromosome aberrations in mouse bone marrow cells when tested *in vivo*. Di(2-ethylhexyl)phthalate, which produced liver tumors, induced a response only in SCEs in CHO cells. Both polybrominated biphenyl (PBB mixture) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) have been shown to demonstrate tumor promotion activity in the two-stage experimental model in rat liver (*23,24*). However, it is not possible to infer retrospectively tumor promotion activity for these chemicals on the basis of the two sex/species results of chronic exposure. Both substances were tumorigenic in the absence of any experimental initiator chemical and in addition to hepatocellular neoplasia, the PBB mixture induced cholangiocarcinomas in female rats and TCDD induced thyroid neoplasia in male rats and female mice. These results suggest that the absence of a positive response in assays for genetic toxicity cannot be used to infer noncarcinogenicity and that the distinction between initiating carcinogens and tumor promoters is possible only in the context of two-stage experimental designs. Previous efforts to distinguish and relate these various effects as the basis of classification of chemicals as genotoxic or nongenotoxic, epigenetic, initiator, promoter, etc. (e.g., *25*), have not achieved a workable acceptance. A detailed discussion of the differences in these classifications is not possible here, but two principal objections are that many chemicals may demonstrate more than one property depending on the circumstances and duration of exposure, and that classifications that are mutually exclusive or depend upon the absence of an observed effect are not useful.

It should be emphasized that the tumorigenicity results used in this evaluation are a product of dose times duration of exposure. Although interim sacrifices were not routinely conducted, the interim death autopsies and incidence of tumor induction at the end of the studies suggests that most of the substances required protracted exposures at or near the maximum tolerated dose (MTD) in order to demonstrate carcinogenic activity. These facts must be contrasted with the relatively limited number of chemicals shown to be tumorigenic with short latent periods following one or a few acute exposures. The majority of these latter substances are direct-acting alkylating agents or are readily metabolized to electrophilic intermediates that are highly reactive with DNA (*26*). The majority of such substances demonstrate clear evidence of genetic toxicity and have been used in the development of most genetic toxicity assays (*1*). Such substances often demonstrate trans-sex/species carcinogenicity. The majority of substances showing the trans-sex/species carcinogenicity in the 2-year rodent studies also showed clear evidence of genetic toxicity, but, as noted, there were some important exceptions. It is possible that the disposition and metabolism of chemicals is an important mitigating factor, even for chemicals that are genotoxic. Such chemicals must gain effective access to DNA in cells in the animal in order to induce genotypic change and this may also be influenced by the dose times duration. However, since there is such a large number of substances that are carcinogenic but do not demonstrate either *in vitro* or *in vivo* genetic toxicity using the tests described here, it is clear that other modes of chemically related carcinogenesis are possible. Evidence from a variety of sources indicate that many chemicals, including those

that demonstrate tumor promotion activity, bring about many alterations in cells that involve nonmutagenic genotypic change, or that are mutagenic at the chromosome level by altering the number of chromosomes (*27*). Such alterations may be related to processes occurring in differentiation and growth or the chromosome segregation apparatus that involve heritable phenotypic changes. It may, therefore, be possible to distinguish the clearly genotoxic chemicals, including aneuploidigens, that have the capacity to induce genotypic heritable change from those that cause adaptive cellular events that lead to heritable phenotypic change. The currently available *in vitro* and *in vivo* genetic toxicity assays can be used effectively to prospectively identify chemicals that have a high probability of acting as inductive carcinogens. The limitations of current assays are that they are unable to distinguish chemicals that may act as adaptive carcinogens from chemicals that are not genotoxic and not carcinogenic. Development of assays that can effectively accomplish the latter depend upon improving our understanding of the processes of differentiation and growth.

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